

Inhibition of Gap Junction and Adherens Junction Assembly by Connexin and A-CAM Antibodies

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Abstract. We examined the roles of the extracellular domains of a gap junction protein and a cell adhesion molecule in gap junction and adherens junction formation by altering cell interactions with antibody Fab fragments. Using immunoblotting and immunocytochemistry we demonstrated that Novikoff cells contained the gap junction protein, connexin43 (Cx43), and the cell adhesion molecule, A-CAM (N-cadherin). Cells were dissociated in EDTA, allowed to recover, and reaggregated for 60 min in media containing Fab fragments prepared from a number of antibodies. We observed no cell-cell dye transfer 4 min after microinjection in 90% of the cell pairs treated with Fab fragments of antibodies for the first or second extracellular domain of Cx43, the second extracellular domain of connexin32 (Cx32) or A-CAM. Cell-cell dye transfer

was detected within 30 s in cell pairs treated with control Fab fragments (pre-immune serum, antibodies to the rat major histocompatibility complex or the amino or carboxyl termini of Cx43). We observed no gap junctions by freeze-fracture EM and no adherens junctions by thin section EM between cells treated with the Fab fragments that blocked cell-cell dye transfer. Gap junctions were found on ~50% of the cells in control samples using freeze-fracture EM. We demonstrated with reaggregated Novikoff cells that: (a) functional interactions of the extracellular domains of the connexins were necessary for the formation of gap junction channels; (b) cell interactions mediated by A-CAM were required for gap junction assembly; and (c) Fab fragments of antibodies for A-CAM or connexin extracellular domains blocked adherens junction formation.

GAP junctions are composed of channels (connexons) that provide intercytoplasmic pathways for the diffusion of small molecules (3, 6). Each channel is thought to be formed by six similar or identical proteins, the connexins (3, 5, 6). The connexins (Cx)¹ comprise a family of proteins with sequence homology and a similar predicted membrane topology (4, 5, 9, 16, 17, 21, 34, 37, 38, 54, 56, 57). The generally accepted topological model for the Cxs has four transmembrane segments with the amino and carboxyl termini located on the cytoplasmic side of the membrane. The transmembrane and extracellular domains are conserved while the cytoplasmic domains, except for the short amino terminus, are structurally diverse. The two highly conserved extracellular domains may be important for the recognition and binding of Cxs on the surfaces of adjacent cells. Therefore, the extracellular domains may play a key role in gap junction assembly and the establishment of cell-cell communication.

We report the first demonstration that Fab fragments of an-

tibodies for the extracellular domains of Cxs impeded cell-cell dye transfer and inhibited the assembly of gap junction structures when added to the media bathing the cells. Our results indicated that functional interactions of the extracellular Cx domains were necessary for the formation of gap junction channels. We also demonstrated that cell interactions mediated by A-CAM (N-cadherin) were required for gap junction assembly between reaggregated Novikoff cells.

We observed that adherens junction formation was inhibited by treatment of the cells with Fab fragments of antibodies for the extracellular domains of Cxs or A-CAM. Adherens junctions are cell-cell membrane specializations that have protein components associated with cadherins extracellularly (50) and with actin filaments on the cytoplasmic surface (47, 48). Anti-A-CAM Fab fragments have previously been reported to alter adherens junction assembly (49). The inhibition of adherens junction assembly by the Fab fragments of antibodies for the Cx extracellular domains was unexpected.

Materials and Methods

Immunoblot Analysis

We isolated Novikoff plasma membranes by a dextran-polyethylene glycol phase separation method (36). The membranes were kept at 4°C in buffer containing 10 mM EDTA (Sigma Chemical Co., St. Louis, MO), 2 mM

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1. *Abbreviations used in this paper:* Cx, connexin; MHC, major histocompatibility complex; MIP, main intrinsic protein; N-CAM, neural cell adhesion molecule.

PMSF (Sigma Chemical Co.), 1 $\mu\text{g}/\text{ml}$ pepstatin (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 1 $\mu\text{g}/\text{ml}$ leupeptin (Boehringer Mannheim Biochemicals) during the entire isolation procedure. The membranes were washed and proteins separated on 10% SDS-polyacrylamide gels (26). The proteins were transferred to nitrocellulose paper (Biotrace NT; Gelman Sciences, Inc., Ann Arbor, MI). The blots were cut into strips, blocked in 3% BSA in TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.4), and probed with the different antibodies. Strips of the transfers were incubated with: (a) a rabbit polyclonal site-directed antibody for the amino terminus of Cx43 (AT-2, see Fig. 1) at 0.5 μg protein/ml that was kindly provided by B. Yancey (California State Polytechnic University, Pomona, CA) (54); (b) an affinity purified rabbit polyclonal antibody for the first extracellular domain of Cx43 (EL-46, see Fig. 1) at 5 $\mu\text{g}/\text{ml}$ (27); (c) an affinity purified rabbit polyclonal antibody for the second extracellular domain of Cx43 (EL-186, see Fig. 1) at 0.5 $\mu\text{g}/\text{ml}$ (27); (d) a rabbit polyclonal antibody for the second extracellular domain of Cx32, residues 164-189 of Cx32 (see Fig. 1), at 1 $\mu\text{g}/\text{ml}$ that was kindly provided by D. A. Goodenough (Harvard University, Cambridge, MA) (17); (e) an affinity purified antibody for the carboxyl terminus of Cx43 (27) at 0.5 $\mu\text{g}/\text{ml}$ (CT-360, see Fig. 1); (f) pre-immune serum for the antibody for the second extracellular domain of Cx32 at 1 $\mu\text{g}/\text{ml}$ that was kindly provided by D. A. Goodenough (17); (g) pre-immune serum as a control for the rabbit polyclonal antibodies or mouse ascites as a control for the mouse mAb; (h) a mouse mAb for the cell adhesion molecule, A-CAM, at 1 $\mu\text{g}/\text{ml}$ (Sigma Chemical Co.) (49); (i) a mouse mAb for a rat major histocompatibility complex (MHC) at 1 $\mu\text{g}/\text{ml}$ (Chemicon International Inc., Temecula, CA); and (j) immune rabbit serum for the main intrinsic protein of the lens (MIP). The blots were washed and reacted with either an alkaline phosphatase-labeled anti-rabbit IgG or an alkaline phosphatase-labeled anti-mouse IgG (Promega Biotec, Madison, WI). The alkaline phosphatase was developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Alkaline Phosphatase Treatment

The Novikoff membranes were washed three times (50 mM Tris-HCl, 10 mM MgCl_2 , 150 mM NaCl) and treated with 20 U/ml of molecular biology grade alkaline phosphatase (3,000 U/mg; Boehringer Mannheim Biochemicals) in buffer containing 0.1% Triton X-100, and 0.05% SDS for 4 h at 37°C (35). Protease inhibitors (2 mM PMSF, 1 $\mu\text{g}/\text{ml}$ pepstatin, and 1 $\mu\text{g}/\text{ml}$ leupeptin) were present in all the solutions used for the alkaline phosphatase treatment. A concurrent sample was treated with alkaline phosphatase in the presence of the alkaline phosphatase inhibitors, 2 mg/ml sodium orthovanadate (Sigma Chemical Co.), 10 mM EDTA, and 10 mM NaH_2PO_4 . Samples were then analyzed by SDS-PAGE (26).

Immunofluorescence

Indirect immunofluorescence was performed on cells attached for 1 h to glass Labtek chamber slides coated with Cell-Tak (BioPolymers Inc., Farmington, CT) according to the manufacturer's instructions. The cells used for anti-Cx antibody labeling were fixed for 10 min in 1% fresh paraformaldehyde, permeabilized with 0.2% Triton X-100 for 10 min, and then blocked and quenched in 3% BSA with 10 mM glycine in PBS (137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) for 30 min. Cells used for anti-A-CAM and anti-MHC labeling were fixed but remained unpermeabilized so that only the cell surface was labeled. The cells were reacted for 2 h at room temperature with nonimmune serum or antibodies for the amino or carboxyl termini of Cx43, anti-MHC, or anti-A-CAM. The samples were washed and incubated for 1 h with goat anti-rabbit or goat anti-mouse FITC-labeled antibodies (Gibco/BRL, Gaithersburg, MD) and viewed with a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Immunogold Labeling for Transmission Electron Microscopy

Pre-embedding Labeling. Isolated Novikoff cell membranes were washed in buffer (1% BSA, 0.05% NP-40, 5 mM EDTA, 0.9% NaCl, and 25 mM Tris base) and incubated for 1 h at room temperature in non-immune serum or with anti-Cx43 antibodies for either the amino or carboxyl termini. The membranes were washed and incubated with 10 nm gold-labeled anti-rabbit IgG (Janssen Life Sciences Products, Piscataway, NJ) at a 1:50 dilution for 1.5 h. The samples were fixed in 1.5% glutaraldehyde with 0.25% tannic acid in 0.1 M cacodylate buffer. The membranes were treated for 1 h with osmium, dehydrated, and embedded in Epon/Araldite.

Post-embedding Labeling. Cells were fixed in 1% paraformaldehyde in

S210 media for 10 min and embedded in Lowicryl K4M at -30°C according to the manufacturer's directions. The K4M was polymerized for 24 h under UV light. Thin sections were blocked in PBS with 1% BSA for 30 min and reacted with non-immune serum or antibodies for the second extracellular domain of Cx43 in PBS (with 1% BSA, 0.1% Tween-20, and 0.1% Triton X-100) for 2–3 h (41). The sections were washed and incubated in 10 nm gold-labeled anti-rabbit IgG (Janssen Life Sciences Products) at a 1:20 dilution for 1.5 h. The grids were washed and stained in 5% uranyl acetate for 30 min.

Morphometric Analysis

We examined thin sections of cell interfaces to evaluate the extent of cell apposition following the antibody Fab fragment treatments. The regions of membrane-membrane contact between adjacent cell pairs were photographed at a magnification of 10,000 and the micrographs were printed at a magnification of 75,000. We measured the membrane-membrane distances between adjacent cell pairs at multiple points along their interfaces to determine the extent of apposition as previously described (53). We also counted the number of adherens junctions present between the cell pairs.

Fab Fragment Production

Fab fragments from the different antibodies were prepared according to the manufacturer's instructions using a Pierce ImmunoPure Fab Preparation Kit (Pierce Chemical Co., Rockford, IL). The antibody Fab fragments were concentrated by centrifugation through Centricon-30 filters (Amicon Corp., Danvers, MA). The protein concentration of the Fab fragments was measured using the bicinchoninic acid protein assay (Pierce Chemical Co.) with BSA as a standard. The papain digestion of the antibodies was checked by separation of the proteins on 13% SDS-polyacrylamide gels which were then silver stained. A prominent band was observed at 55 kD (using non-reducing conditions) with little protein present elsewhere in the gel (data not shown).

Cell Dissociation, Recovery, and Reaggregation

Novikoff cells were cultured as previously reported (22, 33, 39). Cells in logarithmic growth were centrifuged from standard growth media (S210 + 10% newborn calf serum, Gibco/BRL), resuspended in S210 media with 10 mM EDTA at 6×10^5 cells/ml, and placed in a 37°C gyratory shaker incubator (200 rpm) for 15 min. The EDTA treatment was repeated once resulting in >95% single cells. The cells were allowed to recover from the EDTA treatment in S210 media with 5% newborn calf serum (Gibco/BRL) for 90 min (39, 40). Cells were settled onto 35-mm petri dishes (8×10^5 cells per dish) for 60 min in media containing Fab fragments of the various antibodies. Three or more independent sets of experiments were performed with each treatment. Each experimental set was performed by the same person (R. Meyer) on coded samples and without knowledge of sample treatment. Each experimental set included pre-immune serum and at least one other control antibody treatment along with concurrent samples treated with the Fab fragments for the extracellular domains of the Cxs or A-CAM.

Microinjection of Dye

Dye injection studies were performed to evaluate dye permeability between reaggregated cells that had been settled onto petri dishes. One cell of a pair was microinjected with a 4-ms pulse of 1% aqueous Lucifer yellow CH (Sigma Chemical Co.) at 25 psi using a General Valve picospritzer. We monitored cell-cell dye transfer for each cell pair for a 4-min period after dye injection using a Zeiss IM35 inverted microscope (Carl Zeiss). The cell pairs were recorded on videotapes using a Dage SIT camera (Dage-MTI Inc., Wabash, IN), Panasonic video recorder and TV monitor, and on T-Max film (Eastman Kodak Co., Rochester, NY).

Freeze-fracture and EM

Cells were fixed in the petri dishes after dye microinjection by replacing the experimental media with media containing 2.5% glutaraldehyde. The fixed cells were washed and used for freeze-fracture or thin section EM.

We examined freeze-fracture replicas of cell interfaces which were defined as fractured membrane areas comprising at least $57 \mu\text{m}^2$ and containing an indication of cell-cell apposition (39). Cell interfaces were scored for the presence of one or more gap junctions or formation plaques. "Formation plaques" were defined as specialized membrane areas with clusters or arrays of 9–11-nm intramembranous particles (22, 33, 39).

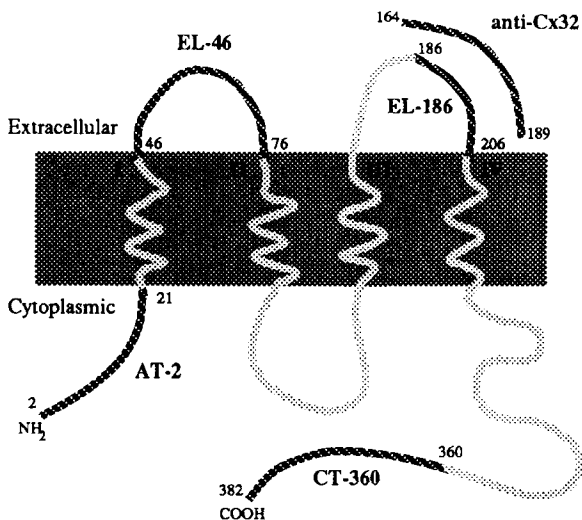


Figure 1. A schematic representation of membrane topology of the polypeptide backbone of Cx43, as deduced from the cDNA sequence (4). The striped regions represent the peptide sequences used to generate the site-directed polyclonal antibodies employed in this study (27, 54). The anti-Cx32 antibody was generated against a segment of Cx32 predicted to be part of the second extracellular domain (17).

Results

We used Novikoff cells as a model system to investigate the components involved in gap junction assembly. The Novikoff cell line was previously derived from a chemically induced rat liver tumor and adapted to growth in suspension culture. The cells are probably not a hepatocyte cell line but may be of Kupffer endothelial cell origin (33). Novikoff cells are readily dissociated without the use of proteases and when

reaggregated form functional junctions over a time course of minutes (22, 39).

Immunoblot Analysis

We used several affinity purified site-directed antibodies to Cx43 (Fig. 1) to characterize a gap junction protein in Novikoff plasma membranes. Membrane proteins were separated with SDS-PAGE and analyzed after transfer onto nitrocellulose paper. The anti-Cx43 antibodies recognized bands with apparent molecular weights of 43 and 45 kD (Fig. 2 A). Antibodies for the amino terminus of Cx43 (AT-2; Fig. 2 A, lane 1) also recognized bands of 29, 49, and 71 kD. We observed differences in the binding of the various anti-Cx43 antibodies to the 43- and 45-kD proteins (Fig. 2 A). The antibodies for the first extracellular domain of Cx43 (EL-46; Fig. 2 A, lane 2) preferentially recognized the 45-kD protein. The antibodies for the second extracellular domain of Cx43 (EL-186; Fig. 2 A, lane 3) recognized both the 43- and 45-kD proteins but reacted more intensely with the 43-kD protein band. The anti-Cx32 extracellular domain antibody (Fig. 2 A, lane 4) and anti-Cx43 carboxyl terminus antibody (CT-360; Fig. 2 A, lane 5) recognized both bands equally. Pre-immune and non-immune serum (Fig. 2 A, lanes 6 and 7) showed no reactivity with Novikoff proteins.

Novikoff plasma membranes were treated with alkaline phosphatase to examine whether the 45-kD protein was phosphatase sensitive. The proteins were separated on SDS-polyacrylamide gels and reacted with the anti-Cx43 antibody for the carboxyl terminus after phosphatase treatment. The antibody detected only the 43-kD band (Fig. 2 B, lane 8) in alkaline phosphatase-treated samples but recognized both the 43- and 45-kD bands in membranes treated with alkaline phosphatase in the presence of alkaline phosphatase inhibitors (Fig. 2 B, lane 9). Our results suggest that the 45-kD band, which was sensitive to alkaline phosphatase, was a

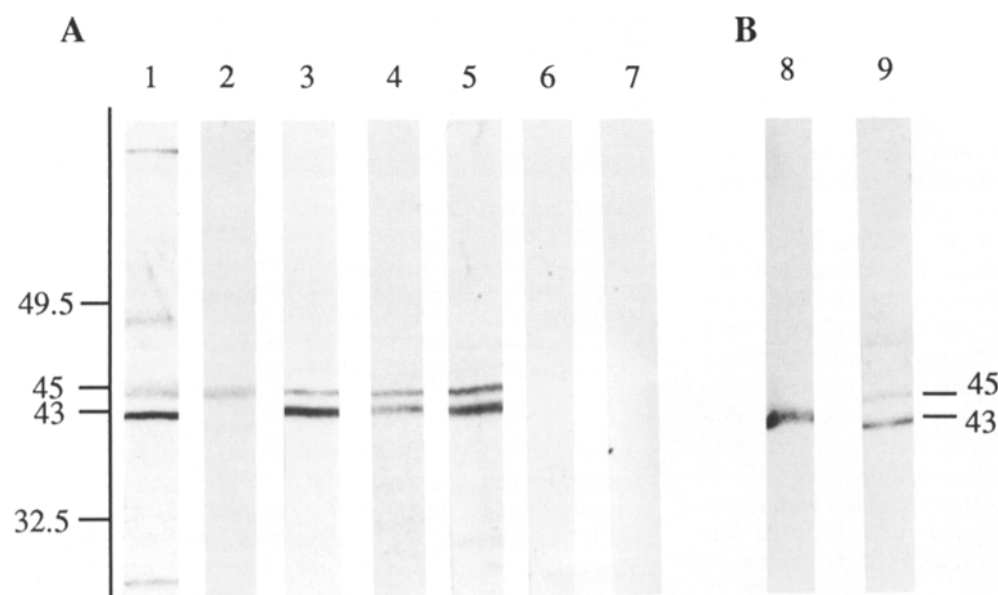


Figure 2. Immunoblots of Novikoff cell membranes that were reacted with anti-Cx antibodies. Plasma membrane proteins were separated on 10% SDS-polyacrylamide gels and analyzed after transfer to nitrocellulose paper. Incubation with several affinity purified site-directed antibodies as well as preimmune serum was followed by reaction with an alkaline phosphatase anti-rabbit IgG according to standard immunoblot conditions. (A) Novikoff membranes probed with: lane 1, antibody for the amino terminus of Cx43 (AT-2); lane 2, antibody for the first extracellular domain of Cx43 (EL-46); lane 3, antibody for the second extracellular domain (EL-186); lane 4, antibody for the second extracellular domain of Cx32; lane 5, antibody for the carboxyl terminus of Cx43 (CT-360); lane 6, preimmune serum for the anti-Cx32 extracellular domain antibody; lane 7, non-immune serum. (B) Novikoff membranes treated with alkaline phosphatase (lane 8) or alkaline phosphatase with orthovanadate and EDTA (lane 9). We probed the transferred proteins with the anti-Cx43 carboxyl terminus antibody (CT-360). Molecular weights are indicated in kD on the left of the figure.

phosphorylated form of Cx43 as reported in other systems (24, 27, 28, 35).

Immunocytochemistry

We used different immunolabeling techniques to demonstrate labeling of the cells with the antibodies used in this study. Indirect immunofluorescence was performed on paraformaldehyde fixed and detergent permeabilized Novikoff cells to examine labeling of the cell with antibodies made to the cytoplasmic domains of Cx43. Negligible levels of immunofluorescence staining were observed on cells treated with non-immune serum (Fig. 3 *a*). We observed discrete punctate immunolabeling at cell-cell interfaces on cells that had been treated with site-directed antibodies to the amino terminus (Fig. 3 *b*) and the carboxyl terminus of Cx43 (Fig. 3 *c*). Punctate immunolabeling was absent from cell surfaces that were not closely apposed. The anti-Cx43 antibodies also reacted with perinuclear regions of the cells.

Paraformaldehyde fixed but unpermeabilized cells were labeled with the anti-A-CAM (N-cadherin) and anti-MHC antibodies to examine their cell surface distribution. We observed diffuse membrane immunolabeling with the anti-A-CAM and the anti-MHC antibodies (data not shown). Negligible levels of immunofluorescence staining were observed on cells treated with mouse ascites fluid as a control for the mAb.

Pre-embedding immunogold labeling and EM were used to localize the anti-Cx43 cytoplasmic domain antibodies to gap junctions in isolated Novikoff membranes (Fig. 4). We observed few gold particles in thin sections of isolated Novikoff gap junction membranes treated with non-immune serum (Fig. 4 *a*). However, strong immunogold labeling was observed on gap junction profiles treated with the anti-Cx43 antibody for the carboxyl terminus (Fig. 4 *b*) or for the amino terminus (data not shown).

An internal control for the specificity of anti-Cx antibodies was the absence of labeling on single membrane vesicles present in the Novikoff membrane preparations. The isolated membranes layered when processed for immunogold labeling such that single membrane vesicles banded in a layer directly above the junction layer. The single membrane vesicles did not label with the anti-Cx43 antibodies for either the carboxyl terminus (Fig. 4 *c*) or amino terminus (data not shown). Therefore, we demonstrated localization of the antibodies to only the gap junction structures.

Post-embedding EM labeling of thin sections of Novikoff cells was performed to localize the anti-Cx extracellular domain antibodies since these domains are inaccessible in gap junctions in whole cell preparations. Novikoff cells were lightly fixed and embedded in Lowicryl at low temperatures to limit denaturation of the connexins and maintain reactivity to the antibodies. Thin sections were incubated with non-immune serum and showed no significant localization of gold particles at sites of cell-cell apposition (Fig. 4 *d*). In contrast, immunogold labeling of thin sections with the anti-Cx43 antibody directed to the second extracellular domain demonstrated antibody reactivity at areas of Novikoff cell-cell appositions (Fig. 4 *e*).

Effects of Antibody Fab Fragments on Gap Junction Assembly

Novikoff cells, which grow as aggregates of cells in suspen-

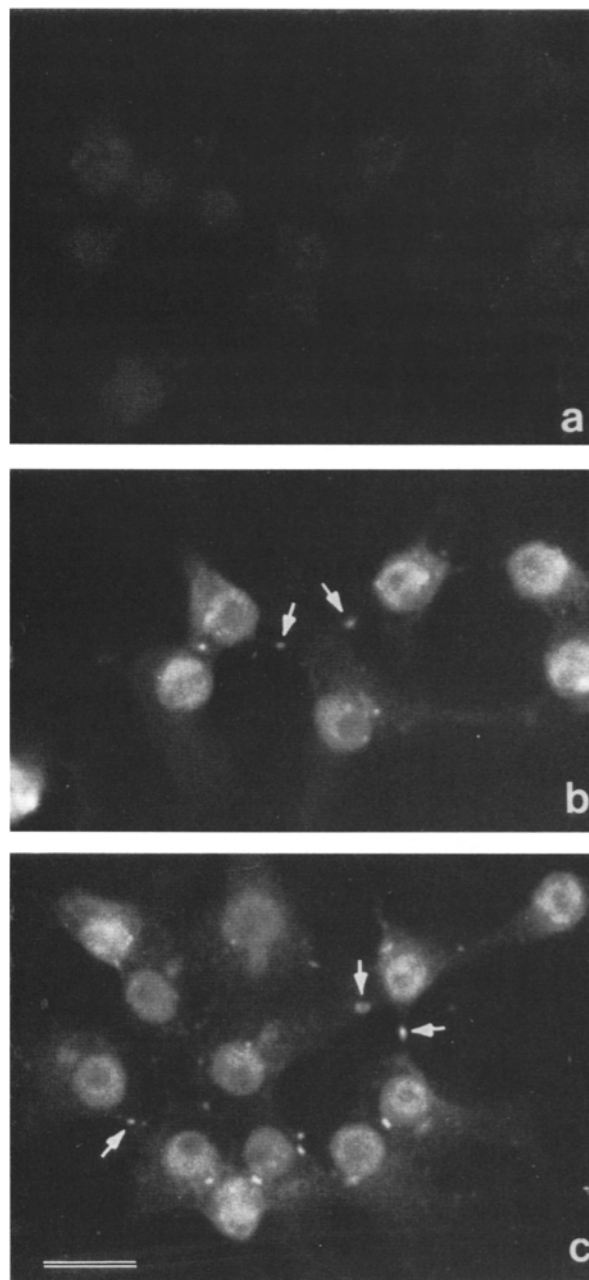


Figure 3. Indirect immunofluorescence of Novikoff cells. Cells were fixed, permeabilized, and reacted with anti-Cx43 antibodies. Little immunofluorescence staining was observed with non-immune serum (*a*), and discrete punctate immunolabeling (arrows) was observed with both the anti-Cx43 antibodies for the amino terminus (AT-2, *b*) and carboxyl terminus (CT-360, *c*). Perinuclear labeling was observed with both the anti-Cx43 antibodies. Bar, 20 μ m.

sion culture, were dissociated with EDTA resulting in >95% single cells. The cells were maintained as single cells in suspension culture for 90 min to allow recovery of the cells from the EDTA treatment. We have previously demonstrated that when cells were dissociated and allowed to recover there was little retention of pre-existing gap junction structures (Meyer, 1991; #56) (39, 40). The cells were allowed to settle onto petri dishes and reaggregated for 1 h in media containing Fab fragments made from the different antibodies. We then assayed for gap junction permeability by microinjection

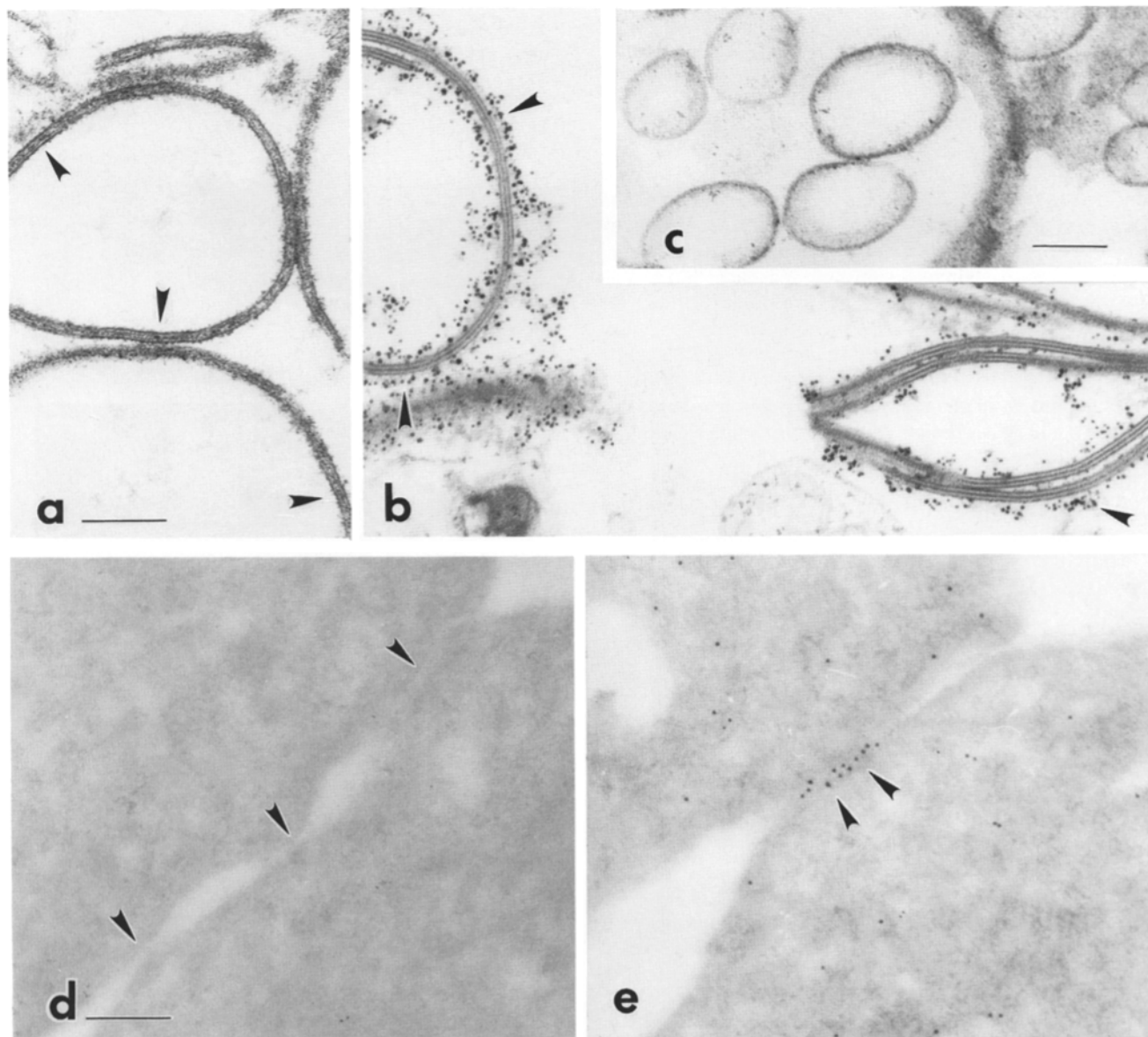


Figure 4. (a, b, and c) Transmission electron micrographs of Novikoff plasma membranes treated with non-immune serum (a) or anti-Cx43 antibody for the carboxyl terminus (b and c). The samples were then incubated with 10 nm gold anti-rabbit IgG, fixed and embedded in Epon-Araldite. The non-immune serum samples contained few gold particles on the gap junctions (a, arrowheads) while the gap junction profiles were strongly immunogold labeled with the antibody for the carboxyl terminus of Cx43 (b, arrowheads). Single membrane vesicles were unlabeled with the anti-Cx43 carboxyl terminus antibody (c). (d and e) Transmission electron micrographs of thin sections of cells embedded in Lowicryl and immunogold labeled. The sections were incubated with non-immune serum (d) or an antibody to the second extracellular domain of Cx43 (e). The non-immune serum showed no gold particles at the sites of cell-cell apposition (d, arrowheads). In contrast, the antibody for the second extracellular domain of Cx43 labeled the site of cell-cell apposition (e, arrowheads). Bars: (a and b) 200 nm; (c) 170 nm; (d and e) 250 nm.

of Lucifer yellow into one cell of a pair. After the microinjection experiments were completed the cell samples were fixed and processed for freeze-fracture and EM to assay for gap junction structures.

Dye Transfer

Control Antibodies. Control Fab fragments included: (a) site-directed antibodies to the amino terminus of Cx43 (Fig. 5 a); (b) site-directed antibodies to the carboxyl terminus of Cx43 (Fig. 5 b); (c) pre-immune serum; (d) non-immune serum; (e) a polyclonal antibody for lens MIP which did not

recognize Novikoff proteins in immunoblots and was used as an immune serum unrelated to connexins; and (f) an anti-rat MHC mAb which recognized a 47-kD protein in Novikoff cells as determined by immunoblots (data not shown). The anti-rat MHC Fab fragments (Fig. 5 c) were added at the highest protein concentration used in the experiments to determine whether simple Fab fragment binding to the cells was sufficient to alter gap junction assembly. We observed cell-cell dye transfer within 30 s after microinjection in all the control antibody Fab fragment treated samples. No effect on the percentage of cell pairs exhibiting dye transfer was observed (Table I).

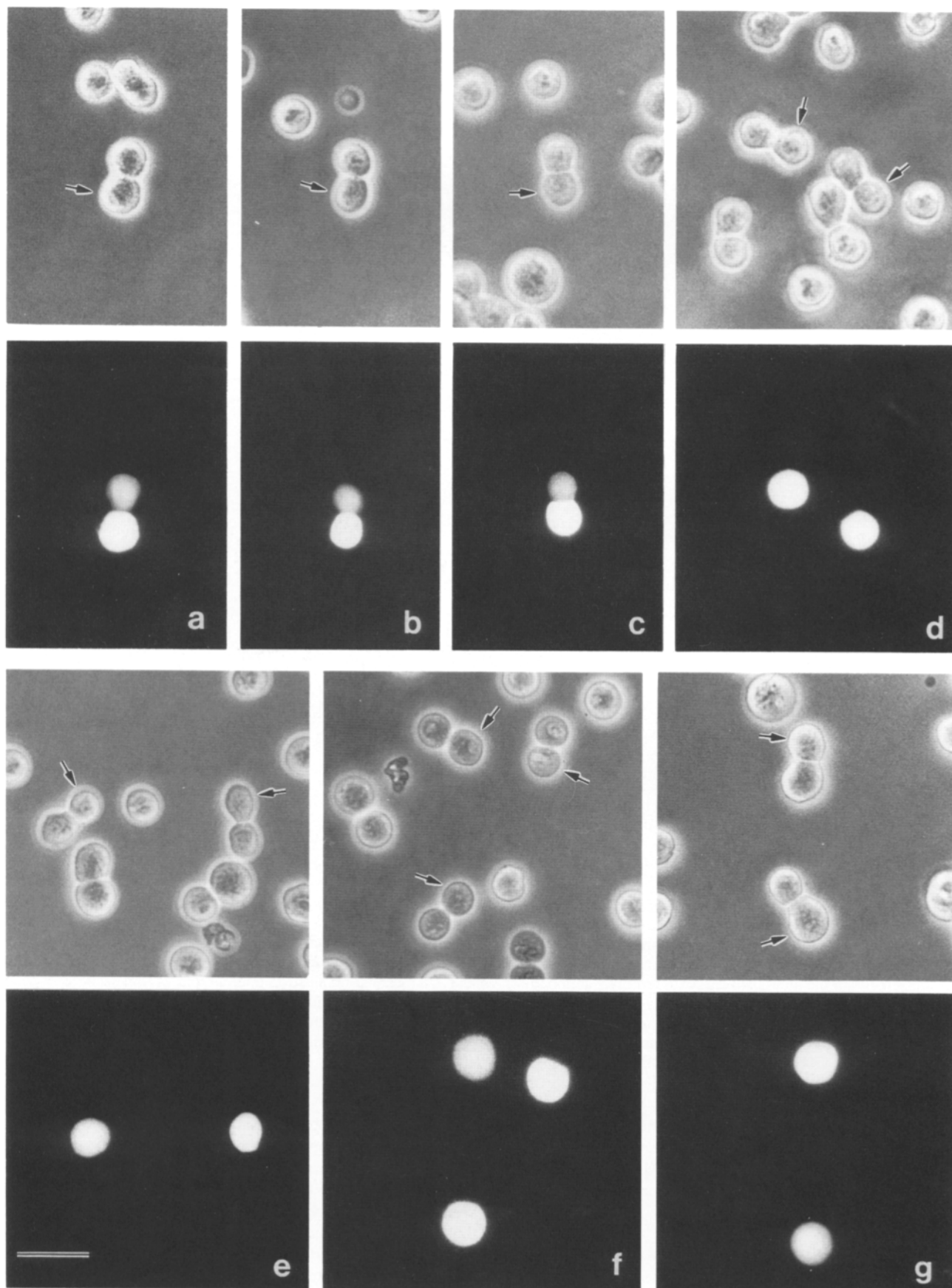


Table I. Antibody Effects on Cell-Cell Dye Transfer

1-h treatment with Fab fragments*	Fab concentration ($\mu\text{g/ml}$)	Cell pairs with dye transfer		Cell pairs without dye transfer after 4 min		Total number of injections
		Number of injections	Percent	Number of injections	Percent	
AT-2	250	40	95	2	5	42
CT-360	120	10	91	1	9	11
	250	52	95	3	5	55
MHC	250	38	91	4	9	42
Non-immune serum	30	12	100	0	0	12
	60	8	100	0	0	8
	120	12	100	0	0	12
	250	13	100	0	0	13
MIP	125	11	92	1	8	12
	250	30	97	1	3	31
Pre-Cx32 164-189	250	18	95	1	5	19
A-CAM	125	6	60	4	40	10
	250	3	7	42	93	45
EL-46	30	15	100	0	0	15
	60	5	12	37	88	42
EL-186	30	7	12	52	88	59
Cx32 164-189	60	8	100	0	0	8
	120	13	100	0	0	13
	250	6	9	60	91	66

* AT-2, Polyclonal antibody specific for the N-terminus of Cx43. CT-360, Affinity purified polyclonal antibody specific for the C-terminus of Cx43. MHC, Monoclonal to the Rat Major Histocompatibility Complex. MIP, Polyclonal antibody to the lens main intrinsic protein. A-CAM, mAb to the α -cell adhesion molecule. EL-46, Affinity purified polyclonal to the first extracellular domain of Cx43. EL-186, Affinity purified polyclonal antibody to the second extracellular domain of Cx43. Cx32-164-189, Polyclonal antibody to the second extracellular domain of Cx32.

Cell Adhesion Molecule Antibody. We examined the role of the cell adhesion molecule, A-CAM (N-cadherin), in gap junction assembly. Antibodies for A-CAM (49) reacted with a 135-kD protein in immunoblots of Novikoff membranes (data not shown). We observed no cell-cell dye transfer 4 min after microinjection in 93% of the cell pairs treated with the anti-A-CAM antibody Fab fragments added at 250 μg protein/ml (Fig. 5 *d*). The percentage of cell pairs exhibiting cell-cell dye transfer was dependent upon the anti-A-CAM Fab fragment concentration (Table I) since only 40% of the cell pairs exhibited cell-cell dye transfer at 125 μg protein/ml.

Antibodies to the Extracellular Domains of Connexins. We evaluated the function of the extracellular domains of connexins in gap junction assembly by treating the cells with Fab fragments of antibodies for the extracellular domains of connexins. The percentage of cell pairs exhibiting cell-cell dye transfer was dependent on Fab fragment concentration.

We observed no cell-cell dye transfer 4 min after microinjection between ~90% of the cell pairs treated with Fab fragments of the antibodies for the first (Fig. 5 *e*) or the second extracellular domains (Fig. 5 *f*) of Cx43 at 30–60 μg protein/ml. The Fab fragments for extracellular domains of Cx43, which were prepared from affinity purified antibodies, only partially blocked dye transfer between cell pairs at lower concentrations (Table I). In contrast, the Fab fragments of antibodies for the second extracellular domain of Cx32 (Fig. 5 *g*) were required at 250 $\mu\text{g/ml}$ to block cell-cell dye transfer. The anti-Cx32 Fab fragments were made from an IgG fraction collected from a protein A column but had not been affinity purified.

Freeze-fracture and Electron Microscopy

The absence of cell-cell dye transfer between 90% of the cell pairs treated with Fab fragments for the extracellular domain

Figure 5. Phase and fluorescence photographs of cell pairs 4 min after one cell of a pair (arrow) was microinjected with Lucifer yellow dye. The reaggregated cells were bathed in media containing Fab fragments of the different antibodies: *a*, antibody for the amino terminus of Cx43 (AT-2); *b*, antibody for the carboxyl terminus of Cx43 (CT-360); *c*, anti-rat MHC mAb; *d*, anti-A-CAM mAb; *e*, antibody for the first extracellular domain of Cx43 (EL-46); *f*, antibody for the second extracellular domain of Cx43 (EL-186); *g*, antibody for the second extracellular domain of Cx32. Bar, 20 μm .

Table II. Antibody Effects on Gap Junction Assembly

1-h treatment with Fab fragments*	Fab Concentration ($\mu\text{g/ml}$)	Interfaces with junctions		Interfaces without junctions		Total number of interfaces
		Number	Percent	Number	Percent	
AT-2	250	15	50	15	50	30
CT-360	250	13	46	15	54	28
MHC	250	14	54	12	46	26
Non-immune serum	250	9	45	11	55	20
Pre-Cx32 164-189	250	16	53	14	47	30
A-CAM	250	0	0	27	100	27
EL-46	60	0	0	28	100	28
EL-186	30	0	0	31	100	31
Cx32 164-189	250	0	0	26	100	26

* AT-2, Polyclonal antibody specific for the N-terminus of Cx43. CT-360, Affinity purified polyclonal antibody specific for the C-terminus of Cx43. MHC, Monoclonal to the Rat Major Histocompatibility Complex. A-CAM, Monoclonal antibody to the α -cell adhesion molecule. Cx32-164-189, Polyclonal antibody to the second extracellular domain of Cx32. EL-46, Affinity purified polyclonal to the first extracellular domain of Cx43. EL-186, Affinity purified polyclonal antibody to the second extracellular domain of Cx43.

of connexin43 or A-CAM could have been due to the closure of the gap junction channels or to inhibition of the assembly of gap junction channels. Therefore, we used freeze-fracture EM to assay for the presence of gap junctions in samples that had been used for dye microinjection. We observed normal appearing gap junctions and formation plaques on $\sim 50\%$ of the cell interfaces between the cells treated with Fab fragments of control antibodies (Table II). The control antibodies included pre-immune serum, non-immune serum, anti-rat MHC, and antibodies for the amino and carboxyl termini of Cx43. Since no gap junctions were present at the start of the reaggregation period (33, 39, 40), the presence of gap junction structures in the control samples indicated that gap junction assembly had occurred (Table II). However, we observed no gap junctions in samples treated with antibody Fab fragments for the first or second extracellular domain of Cx43, the second extracellular domain of Cx32, or the cell adhesion molecule, A-CAM (Table II). We observed a normal distribution of the nonjunction intramembranous particles in all the freeze fracture samples.

Our results suggest that the absence of cell-cell dye transfer between cells treated with Fab fragments for the extracellular domains of connexins or A-CAM was due to the inhibition of gap junction assembly.

Effects of Antibody Fab Fragments on Membrane-Membrane Apposition

Cell-cell adhesion could have been altered by the various antibody Fab fragment treatments. We performed a simple light microscopic assay of cell adhesion to determine whether our results could be explained by cell adhesion differences. We determined the percentages of cells present as singles, pairs, triplets, or clusters of four and more cells following cell reaggregation in the petri dish. No statistically significant difference among the various samples was observed (data not shown). Thus, it is unlikely that our observations were simply due to gross changes in cell adhesion.

We further investigated cell-cell adhesion and the degree of membrane-membrane apposition with transmission EM

and morphometric analysis of thin sections. We counted an average of 2.8 adherens junctions per cell-cell profile between cells treated with the Fab fragments of a control antibody (directed to the cytoplasmic carboxyl terminus of Cx43). An average intermembrane distance of 3 ± 0.4 nm ($n = 53$ cell pairs) was measured in the regions of adherens junctions and a distance of 8 ± 1.1 nm in the nonjunction regions (Fig. 6 a). We observed the same trends of the presence of adherens junctions and close membrane-membrane apposition between cells treated with other control Fab fragments including the pre-immune serum, the anti-Cx43 amino terminus antibody, and the anti-rat MHC antibody (data not shown).

No adherens junctions and a greater intermembrane distance were detected between the cells treated with the anti-A-CAM (N-cadherin) Fab fragments. The intermembrane distance between adjacent cells was 32 ± 1.6 nm (Fig. 6 b; $n = 57$ cell pairs).

We observed no adherens junctions between the cells treated with the antibody Fab fragments for either the first or second extracellular domain of Cx43 or for the second extracellular domain of Cx32. The intermembrane distance between cells treated with Fab fragments of antibodies for the second extracellular Cx domain of Cx43 (Fig. 6 c) was 11.0 ± 1.9 nm ($n = 61$ cell pairs), which was similar to the nonjunction intermembrane distance in control samples. The intermembrane distances between cells treated with Fab fragments for the first extracellular domain of Cx43 and second extracellular domain of Cx32 (data not shown) were also similar to the intermembrane distances for the nonjunction control sample.

Treatment of the cells with Fab fragments for either A-CAM or the extracellular connexin domains blocked gap junction and adherens junction formation, however the modes of action may be different. The cells treated with the anti-A-CAM Fab fragments had increased intermembrane distances between apposing cells compared to controls. In contrast, cells treated with Fab fragments of antibodies for the extracellular domains of connexins had intermembrane distances that were similar to the control sample nonjunction distance.

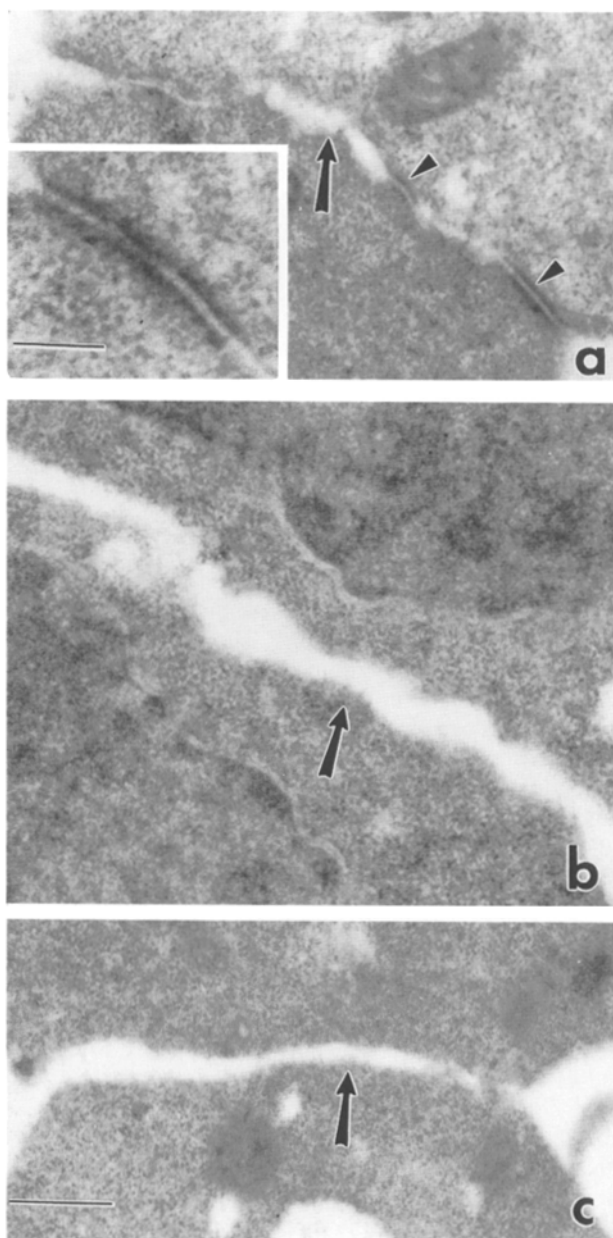


Figure 6. Transmission EM of cell-cell appositions between cells treated with antibody Fab fragments. Cells treated with Fab fragments of antibodies for the carboxyl terminus of Cx43 (*a*) (which allowed dye transfer) were closely apposed (*arrow*) and adherens junctions were present (*arrowheads*). Cells treated with anti-ACAM Fab fragments (*b*) were a considerable distance apart (*arrow*) and no adherens junctions were observed. Cells treated with the Fab fragments of antibodies for the second extracellular domain of Cx43 (*c*) (which blocked cell-cell dye transfer) were only as closely apposed (*arrow*) as the nonjunction distance in the control Fab fragment treated cells (*a*). We observed no adherens junctions in the samples treated with Fab fragments of antibodies for the Cx extracellular domains (*c*). Bar, 55 nm.

Discussion

Little is known about the role the cytoplasmic and extracellular domains of gap junction proteins play in the establishment of cell-cell communication. In this study, we examined the function of the extracellular domains of the Cxs in junc-

tion assembly. Reaggregating Novikoff cells treated with Fab fragments of antibodies directed against the first or second extracellular domains of Cxs showed inhibited cell-cell dye transfer. We observed no gap junction or formation plaques between these cells indicating that the antibody Fab fragments prevented the assembly of gap junction structures in contrast to simple closure of gap junction channels. In addition, cells treated with anti-A-CAM (N-cadherin) Fab fragments displayed inhibited cell-cell dye transfer and the absence of gap junctions. We demonstrated that interactions of the extracellular domains of Cxs and cell interactions mediated by A-CAM are required for the assembly of gap junctions between reaggregated Novikoff cells.

We observed, unexpectedly, that adherens junction formation was inhibited by treatment of the cells with Fab fragments of antibodies for the Cx extracellular domains. Adherens junction assembly was also inhibited by treatment of the cells with Fab fragments of antibodies for A-CAM.

Characterization of Novikoff Gap Junctions

Using immunoblots we demonstrated that four different anti-Cx43 antibodies recognized two major bands (43 and 45 kD) in Novikoff cell membranes. The 45-kD band was alkaline phosphatase sensitive suggesting that the antibodies recognized both phosphorylated and nonphosphorylated forms of the gap junction protein. The differences in antibody binding to the 43- and 45-kD bands that we observed were similar to the differential immunoprecipitation results obtained when the amino and carboxyl termini antibodies were used to immunoprecipitate Cx43 from myocyte cultures (27). The amino terminus antibody also recognized bands of ~29, 49, and 71 kD. The 29-kD band may have been a proteolytic fragment of Cx43 (54). It is not clear whether the 71-kD band was similar to the 70-kD gap junction protein reported in heart (19) or whether it and the 49-kD band represented aggregates of breakdown products and intact Cx43. The lack of reactivity of the 71-kD band with any of the other Cx antibodies, including those for the conserved extracellular domains, suggested that another connexin was not likely to be involved in our experiments.

We showed by pre-embedding immunogold labeling that antibodies for the amino and carboxyl termini of Cx43 labeled typical gap junction profiles as reported by others (17, 19, 27, 46, 54, 55, 57). We used post-embedding labeling of cells to demonstrate localization of antibodies for the extracellular domains of connexins on the Novikoff cell surface in areas of cell-cell contact. Previous reports used pre-embedding methods to immunogold-label gap junctions with anti-Cx extracellular domain antibodies after junctions had been split with hyperosmotic urea or sucrose (17, 34).

Function of the Extracellular Domains of Connexins in Gap Junction Assembly

We examined the role of the extracellular domains of Cxs in gap junction assembly by treating cells with antibody Fab fragments. We report the first demonstration that Fab fragments of antibodies for the extracellular domains of Cxs blocked cell-cell dye transfer and prevented the assembly of gap junction structures. Previous reports have shown inhibited cell-cell dye transfer after microinjection of anti-Cx antibodies into cells (2, 20, 29, 46, 51, 52, 54) or after loading antibodies into permeabilized cells (13). The inhibited

cell-cell dye transfer observed in these previous studies could be due to closure of gap junction channels resulting in a decrease in gap junction permeability. In contrast, we added Fab fragments of antibodies for the extracellular domains of the Cxs to the media containing the cells and prevented gap junction assembly. The external domains, which are thought to protrude extracellularly from the membrane (30), may participate in the initial binding and alignment of the connexons that results in the formation of functional cell-cell channels. Therefore, the antibody Fab fragments may disturb the process of gap junction assembly by interfering with Cx interactions. Synthetic oligopeptides to the extracellular domains of Cxs have also been reported to hinder gap junction formation (8).

We showed that the binding of antibody Fab fragment to the cell surface was not sufficient to alter junction assembly since anti-MHC Fab fragments bound to the cells but did not affect junction formation. Junction assembly was only affected by Fab fragments of antibodies for the Cx extracellular domains and A-CAM suggesting that these are components involved in junction formation.

The only other study that has addressed the function of the extracellular domains of Cxs used paired oocytes expressing Cx32 (7). The investigators observed altered junction permeability when they treated the oocytes with thiol-specific reagents or when they mutated the Cx32 extracellular domain cysteine residues to serine residues. The thiol reagents may have altered the extracellular domain cysteine residues or other cell surface components involved in junction assembly. The mutation of the Cx32 extracellular domain cysteine residues was a more direct test of the extracellular domains function although the study did not demonstrate that the mutated Cxs were present on the cell surface (7).

Relationship of Cell-Cell Contact and Junction Assembly

CAMs are thought to participate in selective cell-cell recognition and adhesion (1, 10, 11, 14, 42, 43, 44) and may be required to initiate a wide variety of cell interactions. Cell-cell adhesion mechanisms are functionally divided into two basic systems, a calcium-independent (the neural cell adhesion molecule; N-CAM) and calcium-dependent systems (the cadherins) (47, 50). N-CAM and the cadherins are large cell surface glycoproteins that are completely different structurally and appear to be evolutionarily unrelated (10). CAM interactions have been proposed to be early recognition events between cells that are necessary for the formation of a variety of specialized intercellular junctions (10, 12, 18, 31, 32, 50).

We investigated the role of A-CAM (N-cadherin) in junction assembly. Anti-A-CAM Fab fragments blocked cell-cell dye transfer and the formation of gap junction and adherens junction structures between reaggregating Novikoff cells. A previous report examined the function of N-CAM by treating cultures of chick neural plate cells with anti-N-CAM Fab fragments (25). A delay in the establishment of gap junction communication was observed. Other investigators transfected cells with cadherin cDNAs to investigate the relationship between cadherins (calcium-dependent CAMs) and gap junction communication (23, 31, 32). Several reports have used S180 cells that were round or spindle shaped with few areas of cell-cell apposition when untransfected (31, 32).

The cadherin cDNA transfected S180 cells formed sheets of epithelioid cells with large areas of cell-cell apposition. Upon addition of anti-cadherin Fab fragments the epithelioid sheets dissociated resulting in cells with shapes resembling the untransfected cells and a concurrent reduction in gap and adherens junctions (31, 32). These investigators proposed that the linkage of the cells by cell adhesion molecules was a necessary event for the expression of junction structures. Since a major change occurred in cell shape and the degree of cell-cell contact, the alterations in junctions that were observed could also be explained by an indirect effect of cell adhesion molecules on the extent and proximity of cell-cell contact. We observed an absence of junctions in Novikoff cells treated with anti-A-CAM Fab fragments without a cell shape change or light microscopically visible change in cell-cell apposition. We did observe an effect on membrane-membrane apposition upon further examination with thin section EM. We suggest that light microscopic observations on cell-cell apposition may not be sensitive enough to detect changes in the degree of membrane apposition.

Adherens junctions represent a specialized class of cell-cell and cell-substrate interactions. The cell-cell adherens junctions are associated with cadherins on the cell surface and the actin containing microfilament system and plaque proteins such as catenin, vinculin, plakoglobin (15, 47), and protein kinases (45) at the cytoplasmic surface. We unexpectedly found that adherens junction assembly was inhibited by treatment of the cells with the Fab fragments of antibodies for the extracellular domains of Cxs as well as A-CAM. Adherens junction assembly had previously only been inhibited by treatment of cells with anti-cadherin Fab fragments (18, 31, 32, 50).

We observed that cells treated with Fab fragments of antibodies for A-CAM were a considerable distance apart. In contrast, the cells treated with the Fab fragments for the extracellular domains of connexins were only as closely apposed as the nonjunction distance in control samples. The mechanism by which Fab fragments of antibodies for the Cx extracellular domains inhibited adherens junction formation is unknown. The anti-Cx Fab fragments could have prevented cell-cell recognition and the acquisition of the very close membrane-membrane apposition normally found within junctions. Alternatively, the antibody Fab fragments may have prevented transmembrane signaling and the activation of cytoplasmic components such as protein kinases (45) thus interfering with adherens junction formation. Further experiments will be necessary to determine whether adhesion per se or the transduction of information arising from cell-cell interactions mediated by cell adhesion molecules is important for junction formation.

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